Reference: *Biol. Bull.* **203:** 338–346. (December 2002)

Biochemical Differences Between Trail Mucus and Adhesive Mucus From Marsh Periwinkle Snails

ANDREW M. SMITH* AND MARTHA C. MORIN

Department of Biology, Center for Natural Sciences, Ithaca College, Ithaca, New York 14850

Abstract. The composition of the adhesive form of marsh periwinkle mucus was compared to the trail mucus used during locomotion. The trail mucus consists primarily of large, carbohydrate-rich molecules with some relatively small proteins. In contrast, the adhesive mucus has 2.7 times as much protein with no significant difference in carbohydrate concentration. The resulting gel has roughly equal amounts of protein and carbohydrate. This substantial increase in protein content is due to the additional presence of two proteins with molecular weights of 41 and 36 kD. These two proteins are absent from the trail mucus. Both proteins are glycosylated, have similar amino acid compositions, and have isoelectric points of 4.75. This change in composition corresponds to an order of magnitude increase in tenacity with little clear change in overall concentration. The difference between adhesive and non-adhesive mucus suggests that relatively small proteins are important for controlling the mechanics of periwinkle mucus.

Introduction

It is generally believed that the fundamental structure of mucous gels consists of giant protein-polysaccharide complexes (Denny, 1983; Davies and Hawkins, 1998), and that mucous secretions can function as effective adhesives due to their viscoelasticity (Grenon and Walker, 1980, 1981). Recent research on limpets, however, has shown that mucus that is used for adhesion can differ substantially from other forms of mucus (Smith *et al.*, 1999). Thus, we investigated the adhesive mucus of the marsh periwinkle *Littorina irrorata*. This snail belongs to a different order of molluscs from limpets, yet it too uses mucus to create a powerful, temporary adhesion. Our goal was to identify specific biochemical characteristics that may be associated with adhesiveness in a mucous gel.

Marsh periwinkles use mucus to glue themselves down during periods of inactivity. During low tide, they forage along the substratum, using a typical, slimy mucous secretion to crawl upon. When the tide comes in, periwinkles migrate up cord grass stems and other marsh vegetation and secrete mucus to glue the lip of their shell to the plant stem. In this way, they can protect themselves from aquatic predators (Warren, 1985; Vaughn and Fisher, 1988). It is possible that the adhesiveness of the mucus results merely from it drying down to a hard film (Denny, 1984). Nevertheless, even when fully hydrated, the mucus used during adhesion creates strikingly stronger attachments than typical molluscan slime. Thus, like limpets (Smith, 1992), periwinkles alternate between active and glued states in a way that suggests a substantial change in the mechanics of their mucus.

There is currently no information on the biochemical structure of periwinkle mucus. As with most mucous secretions, it is probably a dilute gel containing more than 95% water. These gels are usually assumed to resemble mammalian mucus. The organic component of mammalian mucus consists primarily of megadalton-sized, carbohydrate-rich glycoproteins (Silberberg and Meyer, 1982; Allen *et al.*, 1984; Perez-Vilar and Hill, 1999). These fill a large volume and entangle to create a hydrogel. Limpet mucus is quite different, however; it consists of a similarly dilute gel made of much smaller proteins (20 to 220 kDa), only one of which has attached carbohydrate (Grenon and Walker, 1980; Smith *et al.*, 1999). To form a gel, the proteins in limpet mucus may link together through non-covalent bonds. Furthermore, the presence of a specific protein in limpet mucus is correlated with the change from a non-adhesive to an adhesive form (Smith *et al.*, 1999). Comparing periwinkle mucus to these other types of mucus may

Received 16 April 2002; accepted 4 September 2002.

* To whom correspondence should be addressed. E-mail: asmith@ithaca.edu

reveal structural features that are important for adhesion with a mucous-type secretion.

The primary goals of the present study were to determine the general structure of periwinkle mucus, and to see if there are specific features associated with the change to an adhesive mucus. To do this, the composition of adhesive mucus from the marsh periwinkle *Littorina irrorata* was compared to the composition of the normal mucus they use during locomotion, hereafter referred to as trail mucus. In addition, the tenacity of periwinkles using fully hydrated adhesive mucus was measured to confirm that there is a substantial functional change between the adhesive and trail mucus. Because of the unusual nature of these molluscan adhesive secretions, they could provide interesting insights into the structure and function of polymer gels and adhesives.

Materials and Methods

Sample collection

Samples were collected from roughly 200 individuals of the species *Littorina irrorata*. These animals were purchased from Gulf Specimen Marine Laboratories, Inc. (Panacea, Florida) in two separate shipments. They were maintained at 17 °C in a 29-gallon recirculating marine aquarium. The animals normally crawled above the waterline and glued the lip of their shell down at the top of the glass. The process of gluing took several minutes, and involved slowly swiping the entire sole of the foot across the uppermost edge of the shell as the foot retracted into the shell (Bingham, 1972). The resulting thin band of adhesive mucus formed a distinct bridge between the shell and the glass. When the animal was removed, it was easy to scrape this mucus from the glass or the lip of the shell. Trail mucus was collected from the same animals. For these samples, the animals were detached from the aquarium, soaked briefly in seawater, brushed with a paper towel to remove excess moisture, and placed on a glass plate. They were allowed to crawl over the plate for roughly 20 min. The resulting trails were clearly visible, and were scraped off with a clean razor blade. All samples of mucus were dehydrated for 30 min in a rotary Speed-Vac, without heat. Unless otherwise specified, each sample contained 2 to 5 mg dry weight, collected from roughly 20 animals. The snails survived well and continued to produce mucus for over 6 months, but the glue seemed weaker in the later months. Since this implied a possible decrease in quality of the mucus over time, all comparisons between the glue and the trail mucus were made between samples collected at the same time.

Protein and carbohydrate concentration

The protein concentration of 10 dried samples each of adhesive mucus and trail mucus was measured with the bicinchoninic acid (BCA) assay (Pierce Chemical Co.). Several other assay methods were tested, including the Bradford assay (Bradford, 1976) and the ultraviolet absorbance (230 and 280 nm), but these suffered from unacceptable levels of interference from the buffers. In contrast, the BCA assay gave consistent results. Samples to be tested with the BCA assay were typically dissolved in 200–400 μl of 1% sodium dodecyl sulfate (SDS) per milligram of dried mucus. Assuming that half of each sample was dried salt (see results), this gave a mucus concentration of roughly 0.1% to 0.25%. To speed the solubilization, mucus samples were heated at least 10 min in a closed microcentrifuge tube at 80 °C, with occasional vortexing. Even under these conditions, the solution often gelled. In fact, the vortexing usually triggered gel formation. This tendency to gel complicated the assays. To avoid this, the solution was sonicated for less than a minute immediately before the assay. Calibration of the assay was performed with bovine serum albumen in the same buffer.

Several possible sources of error in the protein assay were examined. Though the mucus was mostly soluble in 1% SDS, the presence of a small amount of undissolved material meant that the assays underestimated the total protein. To account for this, the undissolved material from three glue samples and three trail mucus samples was collected by centrifugation. The supernatant was removed, and the pellet was dried in a rotary Speed-Vac. The resulting material was weighed and compared to the mass of the original sample. An additional source of error was that carbohydrates reacted in the BCA assay, slightly inflating the apparent protein concentration. To control for this, different carbohydrates were tested in the assay. Glucose, *N*-acetyl glucosamine, and glucuronic acid were tested separately and together. These carbohydrates gave 2% to 13% of the reaction that a comparable amount of protein gave. Glucuronic acid interfered more strongly than the other two carbohydrates, and the average interference with equal quantities of all three amounted to 8.5%.

The total carbohydrate concentrations of 12 dried samples of adhesive mucus and 9 dried samples of trail mucus were measured using the orcinol-sulfuric acid assay as described by Smith *et al.* (1999). Samples were either dissolved in 1% SDS and diluted to minimize interference, or dissolved directly in 0.1% SDS. Reducing agents were not used as they interfered with the assay. Samples were dissolved as described above. Before dilution, they were vortexed to suspend the remaining fine particles that did not fully dissolve. After addition of the orcinol-sulfuric acid reagent, all the suspended particles rapidly went into solution. Results from dissolution in 0.1% SDS and 1% SDS did not differ. The assay was calibrated with known amounts of glucose dissolved in the same buffer. Since commonly used colorimetric assays for carbohydrates vary in their sensitivity to different carbohydrates (Labare *et al.*, 1989), other standards were tested. These were glucuronic acid, *N*-acetylglucosamine, and potato starch.

Electrophoresis

Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). They were run on discontinuous gels, based on the method of Laemmli (1970) and the detailed protocols of Hames (1990). The gels were 10% acrylamide, and were 10 × 10 cm by 0.75 mm thick. They were stained with Coomassie blue R-250 and photographed with a Kodak EDAS-290 digital imaging system. The software allowed identification of protein bands and quantification of the relative intensity of staining of each band.

Several comparisons were performed. Samples of trail mucus were compared to samples of adhesive mucus (n = 6). In addition, samples dissolved in 2× SDS-PAGE sample buffer (0.125 *M* Tris-Cl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 1.6 *M* urea) were compared to those dissolved in the same buffer without 2-mercaptoethanol (n = 5). Each sample contained mucus pooled from more than 10 snails. Samples were dissolved with heat and sonication as described above, and the same amount of sample was loaded into each lane. To control for possible damage to the samples from the dissolution conditions, some samples were dissolved at room temperature overnight in twice the volume of buffer without sonication or vortexing.

The presence of carbohydrates was determined by periodic acid Schiff (PAS) staining. After electrophoresis of trail mucus and adhesive mucus, the proteins were transferred to nitrocellulose using a semi-dry blotting system (Owl Scientific). Proteins were transferred in a Tris-borate buffer (22.5 m*M* Tris-borate, pH 8.3, 0.1 m*M* EDTA, 0.1% SDS and 25% methanol). Transfer conditions were 300 mA for 3 h. One lane of the transfer was stained with Coomassie blue G-250 (0.005% in 40% methanol), and destained with 40% methanol. The rest of the transfer was stained by PAS following the method of Strömqvist and Gruffman (1992). In addition, four gels were PAS stained directly without transfer, following the method of Zacharius *et al.* (1969). This procedure was performed to detect large carbohydrate-containing molecules in the stacking gel. These may go undetected when a blot is stained because they may not transfer well, and the stacking gel tends to stick to the membrane, interfering with the staining of the blot. The only changes to the published procedure were to double the duration of the incubations with periodic acid and with Schiff's reagent.

The extent of glycosylation was estimated using the method of Segrest and Jackson (1972). This involved running the sample on gels with different total acrylamide concentrations. In a low-percentage gel, proteins with more than 10% carbohydrate will run with a higher apparent molecular weight because the glycosylation inhibits SDS binding. At higher acrylamide concentrations, the effect of sieving determines the mobility more than the charge. Thus, the relative mobility of heavily glycosylated proteins will increase as the gel concentration increases, whereas there will be little effect on proteins with less than 10% carbohydrate. Samples were tested on 5%, 7.5%, 10%, and 15% gels.

Isoelectric focusing

Isoelectric focusing was performed in vertical slab gels. Samples of mucus were dissolved in 50–100 μl of sample buffer (9 *M* urea, 2% CHAPS, and 5% 2-mercaptoethanol) per mg. They were heated at 80 °C in a closed microcentrifuge tube for at least 30 min, and sonicated for less than 1 min. The focusing gels contained the following components: 4.3% total acrylamide with an acrylamide to bis-acrylamide ratio of 37.5:1, 9 *M* urea, 2% ampholytes (pH range 3–10), and 1% Triton X-100. The upper buffer chamber contained 20 m*M* NaOH, and the lower chamber contained 0.085% phosphoric acid. The gels were pre-run at 100 V for 15 min, 200 V for 15 min, then 300 V for 15 min. After the pre-run, the upper buffer was replaced with fresh buffer. The samples were run for 3 h at 300 V. One trial was run for 5 h, yielding the same results to within 0.05 pH units. After each run, a middle lane containing no sample was cut from the gel and sliced into 5-mm strips for determination of the pH gradient. Each of the 5-mm pieces was placed in 1 ml of 0.5% NaCl and left in a sealed test tube overnight with gentle agitation. The next day, the pH of the salt solution was measured with a digital pH meter. The remainder of the gel was stained using the Coomassie blue R-250/$CuSO_4$ method described by Righetti *et al.* (1990). This calibration procedure turned out to be more reliable than using standards, as many standards are not intended for use in gels with urea. The method gave pH as a nearly linear function of distance, with a precision of roughly 0.05 pH units.

Amino acid analysis

Amino acid analysis was performed on the 41 and 36 kD proteins to determine whether or not they had specific features characteristic of mammalian mucin. The method described by Smith *et al.* (1999) was used. Briefly, this involved electrophoresis followed by a tank transfer in tris-borate to a PVDF membrane. Excised bands were hydrolyzed in 6 *M* HCl with 10% phenol and 10% trifluoroacetic acid under vacuum at 150 °C for 40 min. Amino acid compositions were determined on a Beckman System 6300 auto analyzer by the Laboratory for Macromolecular Structure at Purdue University.

Tenacity measurements

The tenacity, defined as attachment force per unit area, of 12 marsh periwinkles was measured when they were using adhesive mucus that had not dried out. This was to confirm

preliminary observations that adhesion involves a substantial difference in the functional properties of the mucus that is not related to the mucus drying. The snails were tested in an aquarium on glass and plastic surfaces. Preliminary observations showed no obvious difference in the tenacity on the two surfaces. The snails were detached primarily by shearing forces. No attempt was made to control for the direction of shear, though, and in some cases there was a significant normal component to the forces. This was because the snails tended to cluster under the lip of the aquarium and on the tubing and pumps, making it difficult to apply the force from a consistent direction. It was clear that detaching in some directions introduced substantial peeling forces, which would weaken the attachment. Nevertheless, the point of this experiment was merely to document a clear change in function from non-adhesive to adhesive rather than to get a precise measure of the tenacity.

Force was measured with a strain gauge force transducer similar to the one described by Smith (1991). The transducer was made of substantially thinner metal, to increase sensitivity, and a half-bridge circuit was used. The transducer was calibrated by hanging known weights from it. The response was linear, and the predictions of the calibration curve differed from the actual values by less than 2% in the range used in this study. Force was measured by connecting an elastic loop around the shell of the snail, connecting this to the transducer, and pulling so that the snails were detached primarily in shear.

The area of contact was estimated from two measurements: the area of shell that is glued down and the area of the mucous patch left on the surface after detachment. The lip of the shell is glued to the substratum only along one edge, resulting in a thin arc of adhesive mucus. The length of this arc was measured by laying a strip of paper marked with fine gradations along the edge of the shell and measuring that part of the lip that was glued down. The length was also estimated by using calipers to measure the mucous patch directly. The width of the mucous arc was estimated by measuring the thickness of the lip of the shell with calipers. The width of the mucous patch was also measured directly. Given the dimensions of the mucous patch and the fact that it is a dilute gel rather than a clearly defined solid, there was significant error in these measurements. For both methods, most of the error was in measuring the thickness, and based on the precision achievable with our measurements, the maximum error was roughly 30%. In some cases, the mucous patch was too distorted to measure. Therefore, since all the snails were the same size and the measurements of the area of adhesion were consistently the same given the limits of precision in the measurements, the same value was used in all the tenacity calculations (3.5 mm^2, based on 7-mm arc length, 0.5-mm width). Finally, as an additional check on the accuracy, the area was estimated from the mass of the collected mucus. The average mass of the samples was 1.8 mg $\pm$ a standard deviation (SD) of 1.0 mg. Assuming that mucus has the same density as water, a 1.8-mg patch of mucus 7 mm long by 0.5 mm wide would be approximately 0.5 mm tall. Visual inspection of the patches suggested that, indeed, the patch was about as tall as it was wide. Note that these estimates of area are likely to give a conservative estimate of tenacity because the mucus is stretched thinner between the shell and substratum during normal attachment.

After each tenacity measurement, the mucus was collected by scraping with a clean, dry razor blade and placed into a fresh microcentrifuge tube. The samples were immediately weighed on a digital balance to the nearest 0.1 mg. Then the amount of carbohydrate in each sample was determined using the orcinol-sulfuric acid assay. The total organic concentration was estimated assuming that the ratio of protein to carbohydrate was the same as measured in experiments described above.

For comparison, the shear tenacity of 23 active periwinkles was measured. This provided an estimate of the tenacity due to the trail mucus. Force was measured as described above. The area of the foot in contact with the substratum was calculated from the length and width of the foot as measured with calipers. The foot was assumed to have an elliptical outline. Note that this measurement will overestimate the tenacity due to the trail mucus alone, since a snail may use an active process such as suction to hold its foot down. In contrast, no such active process can augment the tenacity of a snail using adhesive mucus, since the foot is withdrawn into the shell. Furthermore, the band of adhesive mucus between the shell and substratum is often stretched in a way that weakens the adhesion, while the mucus under the foot of an active snail is in a thin layer, which would provide better adhesiveness. Thus, the difference between trail mucus and adhesive mucus is probably larger than estimated by these measurements.

Results

Protein and carbohydrate concentration

The compositions of the adhesive mucus and the trail mucus differed significantly even when both were fully dehydrated (Fig. 1). The primary difference was that the adhesive mucus had 2.7 times as much protein as the trail mucus (Student's t test, $P < 0.001$). In contrast, the carbohydrate concentrations were not significantly different ($P = 0.15$). It is worth noting that the samples used in this comparison were from animals that had been in an aquarium for over 2 months. Several glue samples taken from recently collected animals had higher protein and carbohydrate concentrations. The same may have been true of the trail mucus, though this was not tested. The protein content of dried adhesive mucus from freshly collected animals was

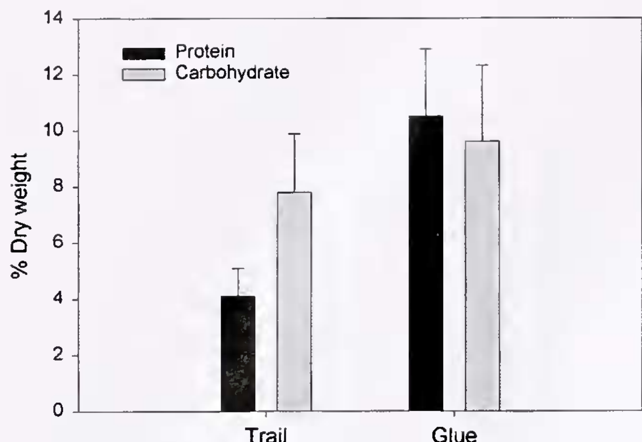

Figure 1. Comparison of the protein and carbohydrate content by dry weight of the two forms of periwinkle mucus. Six to ten pooled samples of each type were tested, with each pooled sample containing mucus from about 10 snails.

15.8% ± 2.5% (Mean ± SD, n = 4). The carbohydrate concentration was 17.1% ± 3.1% (n = 5).

The remainder of the dried sample was presumably inorganic material left over from evaporating seawater. Previous research on other marine mucous secretions found that roughly 50% of the dry weight was inorganic residue (Connor, 1986; Davies *et al.*, 1990). This figure can also be estimated from the percentage of inorganic salts in seawater (3.56%, Schmidt-Nielsen, 1990); for a typical marine mucus containing 96%–98% water, we would predict that inorganic material would make up 46%–64% of the dry weight.

The protein concentration is likely to be an underestimate. Since reducing agents could not be used in the protein assay, the solubilization was not total. The dried insoluble material amounted to 13% ± 7% of the total dry weight (n = 6), with similar results for trail mucus and glue. This undissolved material would not be detected by the assay. This error would only be partially offset by the slight increase in absorbance due to carbohydrate reacting with the assay. The interference from carbohydrates probably led to the protein values being as much as one percentage point above their actual value (*i.e.*, a calculated value of 11% may have actually been 10%, and 4% may have been 3%).

The value for carbohydrate content is also an underestimate. This is primarily due to differential sensitivity of the orcinol assay to different sugar derivatives. The calibration was based on glucose, so it should reflect the neutral sugars accurately. There were no significant differences between the assay's response to glucose and its response to glucuronic acid or starch (Student's t test, P = 0.49 and 0.07 respectively). Thus, the assay should accurately account for uronic acid content, and it should not be significantly affected by degree of polymerization. The assay did not detect N-acetyl glucosamine though; 0–20 μg of this sugar produced absorbances that were within the normal variation of the blanks. Because amino sugars are common components of polysaccharides, and since many oligosaccharides are linked to proteins *via* amino sugars, they are likely to be present in mucus in significant quantities. In three studies of mucus-like gels from marine invertebrates, the quantity of amino sugars was equal to roughly half the neutral sugar (Grenon and Walker, 1980; Meikle *et al.*, 1988; Flammang *et al.*, 1998). Thus, the carbohydrate concentrations may be 50% greater than the values reported here. Finally, the presence of strong acid ensured that, unlike the protein assays, this assay did not have a problem with solubilization.

In the process of dissolving the samples it was noted that the adhesive mucus had different mechanical properties from the trail mucus. The most striking difference was that the trail mucus did not gel under the conditions used to dissolve samples for these assays. In contrast, the adhesive mucus formed gels ranging from viscous solutions to firm gels that did not flow when tipped upside down. At concentrations higher than 1 mg of dry material per 200 μl of buffer (*i.e.*, roughly 0.25% organic material), it was difficult to pipette. Furthermore, the dissolved mucus stuck to the pipet tip or any surface that it contacted, leaving fine, cobweb-like threads when the tip was pulled away.

Electrophoresis

SDS-PAGE showed a clear difference between the protein compositions of adhesive mucus and trail mucus. The protein component of the adhesive mucus consisted almost entirely of a pair of proteins with molecular weights of 41 and 36 kD (Fig. 2). An analysis of digital images indicated that these proteins made up more than 95% of the total

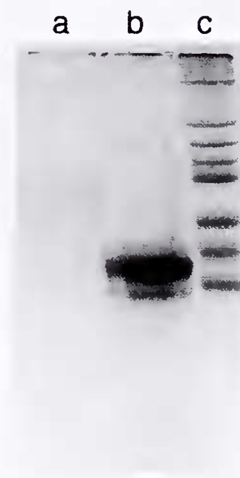

Figure 2. SDS-PAGE comparison of marsh periwinkle trail mucus (a) and adhesive mucus (b). Molecular weight markers are in lane c and have the following molecular weights: 205, 116, 97, 84, 66, 55, 45, and 36 kD. Both forms of mucus were fully dissolved by heating in the sample buffer.

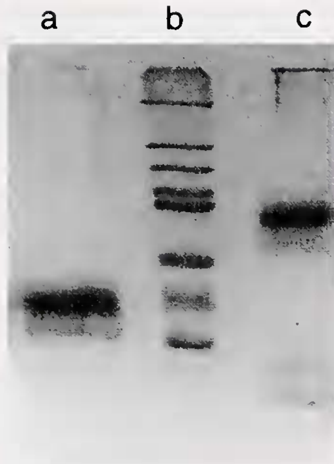

Figure 3. The effect of reducing agents on the proteins found in adhesive mucus. The sample in lane a was reduced prior to running on the gel, while the sample in lane c was not reduced. Molecular weight markers are in lane b and have the following weights: 205, 116, 97, 84, 66, 55, 45, and 36 kD.

protein in the adhesive, yet they were not present in the trail mucus. The proteins found in the trail mucus were different from those found in the adhesive mucus. The main proteins were 65, 63, and 59 kD (Fig. 2). Bands in this range occasionally showed up in the adhesive mucus, but only in relatively small amounts. In both forms of mucus, there was no material at the top of or within the stacking gel, and there were only a few faint bands near the top of the resolving gel. Finally, the overall difference in protein concentration that was detected by the BCA assay was also apparent in the stained gels.

Disulfide bonds affect the structure of the 41 and 36 kD proteins. When these bonds are not broken, the two primary bands show up at 64 and 59 kD (Fig. 3). Thus, disulfide bonds may change the protein structure or SDS binding to create a greater apparent molecular weight. Alternatively, the 41 and 36 kD proteins may be subunits of slightly larger proteins that are held together by disulfide bonds. The key finding, though, is that the proteins are not linked into giant complexes, unlike mammalian mucin. With disulfide bonds intact, there were faint bands at a lower molecular weight, but no significant bands above 64 kD in the resolving or stacking gel.

PAS staining showed that most of the carbohydrates in the mucus appear to be in the form of very large molecules (Fig. 4). There was distinct staining in the stacking gel or at the interface between the stacking and resolving gels. There was faint staining at the position of the 41 kD protein, but it was barely visible. Visual inspection of four stained gels showed no consistent difference between the amount of the large carbohydrates in the adhesive and trail mucus (Fig. 4). It is also possible that there were larger carbohydrate-containing molecules that could not even enter the gel. Finally, PAS staining of transfers showed that the 41 and 36 kD proteins and the trail mucus proteins were glycoproteins, but there was not enough carbohydrate to detect consistently with the in-gel staining.

When samples of adhesive mucus were run on gels with different acrylamide concentrations, there was no difference in the apparent molecular weight of the proteins. This is consistent with the previous results showing that the 41 and 36 kD proteins are not heavily glycosylated. On a 5% gel, all proteins less than 55 kD ran with the tracking dye. On 7.5%, 10%, and 15% gels, the proteins ran at 42.5, 43.5, and 41.5 and at 36, 35.5, and 36 respectively.

Isoelectric focusing and amino acid analysis

All of the proteins in the mucus were acidic. The 41 and 36 kD proteins focused as a single band at a pI of 4.75. The proteins in trail mucus focused as two bands with pI values of 4.91 and 5.15. Because of the low concentrations, the similar molecular weights and isoelectric points, and the format of the gels, 2-dimensional electrophoresis was not performed. Thus, it is uncertain which of the proteins in trail mucus corresponded to which isoelectric points.

The amino acid compositions of the 41 and 36 kD proteins were similar to each other (Table 1). As calculated using the method of Marchalonis and Weltman (1971), the relatedness value between the proteins was 30; any value below 100 suggests relatedness. Proline, serine, and threonine make up 18% and 20% of the amino acids in these two proteins; in comparison, these typically constitute 40% of the amino acids in mammalian mucin (Allen *et al.*, 1984).

Figure 4. A PAS-stained polyacrylamide gel comparing the adhesive mucus (a) to the trail mucus (b). The arrowhead marks the expected location of the 41 kD protein, based on a lane stained with Coomassie blue. The horizontal line marks the boundary between the stacking and resolving gels.

Table 1

Amino acid compositions of the proteins found in adhesive periwinkle mucus (values in residues per thousand)

Amino acid	41-kD protein	36-kD protein
ASX	85	78
GLX	67	55
SER	51	56
GLY	72	42
HIS	24	12
ARG	63	59
THR	96	73
ALA	70	68
PRO	55	54
TYR	57	65
VAL	72	74
MET	29	42
ILE	49	64
LEU	70	88
PHE	61	77
LYS	80	91

Tenacity measurements

The shear tenacity of marsh periwinkles using adhesive mucus was dramatically stronger than the shear tenacity of active periwinkles with trail mucus under their feet. This was true even though the adhesive mucus was fully hydrated. The average tenacity using adhesive mucus was 73 $\pm$ 43 kPa ($n = 12$). The highest tenacities were 130 to 140 kPa. These tended to occur when the detaching force applied little peeling component. The average carbohydrate concentration of the adhesive mucus that created these tenacities was only 1.0% $\pm$ 0.4% (w/w, $n = 11$). One of the samples was lost because it detached with the snail and ended up underwater. In contrast, the average shear tenacity of active periwinkles was 6.6 $\pm$ 3.1 kPa ($n = 23$). This is comparable to data reported for other periwinkle species by Davies and Case (1997).

Discussion

The mucus used by marsh periwinkles for glue is distinctly different from the mucus used for locomotion. As is the case for limpets (Smith *et al.*, 1999), the key difference correlating with adhesion is the presence of one or two specific proteins. For marsh periwinkles, the change is much more striking than it is with limpets; the additional protein amounts to roughly half of the organic material in the mucus, while the trail mucus has relatively little protein. This correlation strongly suggests a role for these proteins in making the mucus adhesive.

In order to understand the function of these proteins, it is necessary to consider the biochemical structure of marsh snail mucus. Unlike limpet mucus, carbohydrates make up a substantial fraction of the material. The results from the assays on dried marsh periwinkle glue from recently collected animals were 16% protein and 17% carbohydrate. When the undissolved material in the protein assay, the error in the protein assay due to carbohydrates, and the undetected amino sugars are accounted for, it is likely that protein and carbohydrate each make up 20%–25% of the dried adhesive mucus, with the other 50%–60% being inorganic residue. The trail mucus has significantly less protein.

In both forms of mucus, most of the carbohydrates appear to be in the form of large polysaccharides. The carbohydrate assays and the PAS-stained gel showed no noticeable difference between the adhesive and trail mucus in the quantity of these carbohydrates. There was no protein staining associated with these carbohydrates, though the presence of a small amount of protein, as in proteoglycans, has not been ruled out. While it is possible that the carbohydrates at the top of the gel were actually smaller but had limited mobility because they were relatively neutral, this seems unlikely given what is known of gel structure and mucus biochemistry. Finally, only a relatively minor fraction of the carbohydrates are covalently linked to the smaller proteins seen in the adhesive mucus.

A reasonable model for the structure of trail mucus is a dilute, viscoelastic gel consisting primarily of giant carbohydrate-rich molecules. This is typical of most mucous secretions (Denny, 1983; Davies and Hawkins, 1998). The adhesive mucus appears to have a similar amount of giant carbohydrate-rich molecules, but in addition has substantial quantities of relatively small 41 and 36 kD proteins. This suggests that the 41 and 36 kD proteins cause the functional change, perhaps by changing the mechanics of the gel. For example, these proteins could cross-link with each other, with the carbohydrates, or both. Such cross-links would substantially affect the mechanics of the secretion (Denny, 1983). It is also possible that the large carbohydrates in the adhesive mucus have structural differences from those in the trail mucus, though this was not studied. For example, the uronic acid content or the degree of sulfation may differ. This leads to two primary hypotheses. First, the adhesive mucus may be a modified version of the trail mucus, with the 41 and 36 kD proteins added. Alternatively, the large carbohydrates in adhesive mucus may differ as well, which may contribute to adhesion. For example the carbohydrates may be a type that is more capable of interacting with each other or the 41 and 36 kD proteins. In either case, the striking change in protein content argues that these proteins play a key role. The relative importance of smaller, cross-linked proteins makes sense for an adhesive. It is desirable for many adhesives to be made of smaller polymers that can flow more readily to achieve intimate contact with a surface, then cross-link into a stiff network (Bikerman, 1958; Wake, 1982; Waite, 1983). The proteins found in the trail mucus may also play a role in the mechanics of that gel, though

they are not present in a large quantity. The isoelectric point of these proteins falls in the range found for the 41 and 36 kD proteins and the proteins from limpet mucus (Smith *et al.*, 1999), suggesting that there may be some similarity among these proteins.

The change in the composition of mucus corresponds to a substantial functional change. The switch to the adhesive form of mucus increases the shear tenacity a full order of magnitude, from roughly 7 to 73 kPa. Furthermore, the difference is probably greater than these data indicate. The tenacity of snails using adhesive mucus is likely to be an underestimate, due to peeling and the conservative estimate of area that was used. It is likely that tenacities commonly exceed 100 kPa, as is seen with limpets (Smith, 1991, 1992). In contrast, the tenacity of active snails gives an overestimate of the adhesiveness of trail mucus, since much of the adhesive force at that time probably results from suction. This was the case for limpets (Smith, 1991, 1992).

This large increase in tenacity is achieved without concentrating the mucus appreciably. As measured by the orcinol assay, only 1% of the mass of the adhesive mucus was carbohydrate. Adding in carbohydrates that may have been undetected and protein, the total organic concentration of the adhesive mucus was roughly 2%–3% (w/w). This concentration is typical for normal mucous secretions. Thus, the adhesion is not due to changes in mucus concentration; it is more likely due to the differences in composition.

It should not be surprising that different forms of mucus have different compositions and different mechanical properties. There is wide variation in the gross composition of mucous secretions (Davies and Hawkins, 1998), and in their function (Denny, 1989). For example, some mucous secretions have roughly 10 times as much carbohydrate as protein, while in others the reverse is true (Meikle *et al.*, 1988; Davies and Hawkins, 1998; Smith *et al.*, 1999). The differences in function are similarly dramatic: some mucous secretions are designed for lubrication, whereas others are powerful adhesives. Furthermore, it is not unusual for a mollusc to have as many as 10 types of secretory cell on the epithelium of its foot (*e.g.*, Grenon and Walker, 1978). Given these facts, we have no reason to expect that all invertebrate mucous secretions will depend solely on the same type of giant glycoproteins.

There are probably a number of types of mucous gel. Meikle *et al.* (1988) found substantial differences between the mucus of six coral species. Some produce a carbohydrate-rich mucus consisting of megadalton-size glycoproteins with an amino acid composition dominated by serine and threonine, like mammalian mucin. Other corals produce a protein-rich mucus consisting of much smaller subunits (around 150 kD) whose amino acid composition has a high proportion of acidic amino acids. This resembles limpet mucus. Finally, some corals produce a mucus that appears to be dominated by large polysaccharides with very little protein. This may be similar to the mucus of the neogastropod *Nucella emarginata*, which contains 25% carbohydrate by dry weight and only 1% protein (Connor, 1986). The trail mucus produced by marsh periwinkles may also resemble this, whereas the adhesive mucus also contains smaller proteins that are superficially similar to those found in limpet mucus.

As Davies and Hawkins (1998) point out, relatively little is known about the structure of invertebrate mucous secretions. In particular, they note that almost no work has been done linking biochemical structure to function. Our research on marsh periwinkles provides a substantial step forward in this area. There appear to be different categories of mucus. Typical lubricating mucous gels whose mechanics depend solely on giant protein-polysaccharide complexes have been well studied. We have identified different molecules that may be used in situations where greater mechanical strength is needed. These relatively small, acidic proteins from limpet and periwinkle mucus are likely to have interesting structural and chemical properties.

Acknowledgments

We thank S. Johnsen, W. M. Kier, and J. H. Waite for helpful comments. We also thank P. Cowley for assistance with the tenacity measurements. This work was supported in part by an Ithaca College Summer Grant for Faculty Research to AMS.

Literature Cited

Allen, A., D. A. Hutton, J. P. Pearson, and L. A. Sellers. 1984. Mucus glycoprotein structure, gel formation and gastrointestinal mucus function. In *Mucus and Mucosa*, J. Nugent and M. O'Connor, eds. *Ciba Found. Symp.* **109:** 137–156.

Bikerman, J. J. 1958. The rheology of adhesion. Pp. 479–503 in *Rheology: Theory and Applications*, Vol. 3, F. R. Eirich, ed. Academic Press, New York.

Bingham, F. O. 1972. The mucus holdfast of *Littorina irrorata* and its relationship to relative humidity and salinity. *Veliger* **15:** 48–50.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

Connor, V. M. 1986. The use of mucous trails by intertidal limpets to enhance food resources. *Biol. Bull.* **171:** 548–564.

Davies, M. S., and C. M. Case. 1997. Tenacity of attachment in two species of littorinid, *Littorina littorea* (L.) and *Littorina obtusata* (L.). *J. Molluscan Stud.* **63:** 235–244.

Davies, M. S., and S. J. Hawkins. 1998. Mucus from marine molluscs. *Adv. Mar. Biol.* **34:** 1–71.

Davies, M. S., H. D. Jones, and S. J. Hawkins. 1990. Seasonal variation in the composition of pedal mucus from *Patella vulgata* L. *J. Exp. Mar. Biol. Ecol.* **144:** 101–112.

Denny, M. W. 1983. Molecular biomechanics of molluscan mucous secretions. Pp. 431–465 in *The Mollusca*, Vol. 1, K. Wilbur, K. Simkiss, and P. W. Hochachka, eds. Academic Press, New York.

Denny, M. W. 1984. Mechanical properties of pedal mucus and their consequences for gastropod structure and performance. *Am. Zool.* **24:** 23–36.

Denny, M. W. 1989. Invertebrate mucous secretions: functional alternatives to vertebrate paradigms. In *Mucus and Related Topics,* E. Chantler and N. A. Ratcliffe, eds. *Symp. Soc. Exp. Biol.* **43:** 337–366.

Flammang, P., A. Michel, A. V. Cauwenberge, H. Alexandre, and M. Jangoux. 1998. A study of the temporary adhesion of the podia in the sea star *Asterias rubens* (Echinodermata, Asteroidea) through their footprints. *J. Exp. Biol.* **201:** 2383–2395.

Grenon, J. F., and G. Walker. 1978. The histology and histochemistry of the pedal glandular system of two limpets, *Patella vulgata* and *Acmaea tessulata* (Gastropoda: Prosobranchia). *J. Mar. Biol. Assoc. UK* **58:** 803–816.

Grenon, J. F., and G. Walker. 1980. Biomechanical and rheological properties of the pedal mucus of the limpet, *Patella vulgata* L. *Comp. Biochem. Physiol. B* **66:** 451–458.

Grenon, J. F., and G. Walker. 1981. The tenacity of the limpet, *Patella vulgata* L.: an experimental approach. *J. Exp. Mar. Biol. Ecol.* **54:** 277–308.

Hames, B. D. 1990. One dimensional polyacrylamide gel electrophoresis. Pp. 1–147 in *Gel Electrophoresis of Proteins: A Practical Approach,* B. D. Hames and D. Rickwood, eds. IRL Press, Oxford.

Labare, M. P., K. Guthrie, and R. M. Weiner. 1989. Polysaccharide exopolymer adhesives from periphytic marine bacteria. *J. Adhesion Sci. Technol.* **3:** 213–223.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Marchalonis, J. J., and J. K. Weltman. 1971. Relatedness among proteins: a new method of estimation and its application to immunoglobins. *Comp. Biochem. Physiol. B* **38:** 609–625.

Meikle, P., G. N. Richards, and D. Yellowlees. 1988. Structural investigations on the mucus from six species of coral. *Mar. Biol.* **99:** 187–193.

Perez-Vilar, J., and R. L. Hill. 1999. The structure and assembly of secreted mucins. *J. Biol. Chem.* **274:** 31751–31754.

Righetti, P. G., E. Gianazza, C. Gelfi, and M. Chiari. 1990. Isoelectric focusing. Pp. 149–216 in *Gel Electrophoresis of Proteins: A Practical Approach,* B. D. Hames and D. Rickwood, eds. IRL Press, Oxford.

Schmidt-Nielsen, K. 1990. *Animal Physiology: Adaptation and Environment.* Cambridge University Press, Cambridge. 607 pp.

Segrest, J. P., and R. L. Jackson. 1972. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Pp 54–63 in *Methods in Enzymology,* Vol 28B, V. Ginsburg, ed. Academic Press, New York.

Silberberg, A., and F. A. Meyer. 1982. Structure and function of mucus. In *Mucus in Health and Disease II,* E. N. Chantler, J. B. Elder, and M. Elstein, eds. *Adv. Exp. Med. Biol.* **144:** 53–74.

Smith, A. M. 1991. The role of suction in the adhesion of limpets. *J. Exp. Biol.* **161:** 151–169.

Smith, A. M. 1992. Alternation between attachment mechanisms by limpets in the field. *J. Exp. Mar. Biol. Ecol.* **160:** 205–220.

Smith, A. M., T. J. Quick, and R. L. St. Peter. 1999. Differences in the composition of adhesive and non-adhesive mucus from the limpet *Lottia limatula. Biol. Bull.* **196:** 34–44.

Strömqvist, M., and H. Gruffman. 1992. Periodic acid/Schiff staining of glycoproteins immobilized on a blotting matrix. *Biotechniques* **13:** 744–749.

Vaughn, C. C., and F. M. Fisher. 1988. Vertical migration as a refuge from predation in intertidal marsh snails: a field test. *J. Exp. Mar. Biol. Ecol.* **123:** 163–176.

Waite, J. H. 1983. Adhesion in byssally attached bivalves. *Biol. Rev. Camb. Philos. Soc.* **58:** 209–231.

Wake, W. C. 1982. *Adhesion and the Formulation of Adhesives.* Applied Science Publishers, London. 332 pp.

Warren, J. H. 1985. Climbing as an avoidance behaviour in the salt marsh periwinkle, *Littorina irrorata* (Say). *J. Exp. Mar. Biol. Ecol.* **89:** 11–28.

Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **30:** 148–152.